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KONINKRIJK DER NEDERLANDEN

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This is to declare that in the Netherlands on December 24, 2003 under No. PCT/NL03/00930, in the name of:

DE STAAT DER NEDERLANDEN, VERTEGENWOORDIGD DOOR DE MINISTER VAN VOLKSGEZONDHEID, WELZIJN EN SPORT

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in Utrecht, the Netherlands (NL)

an international patent application was filed for:

"A respiratory syncytial virus with a genomic defiency complemented in trans",

and that the documents attached hereto correspond with the originally filed documents.

Rijswijk, February 4, 2005

In the name of the president of the Netherlands Industrial Property Office

Mrs. C.M.A. Streng

A Respiratory Syncytial Virus with a genomic deficiency complemented *in trans*

Field of the invention

5   The present invention relates to the field of vaccination, and more specifically to vaccines against disease caused by pneumoviruses such as e.g. Respiratory Syncytial Virus (RSV). The invention pertains RSV virions carrying an RSV genome in which a gene that is essential for infectivity has been inactivated, while the corresponding wild type gene-product is complemented *in trans* to the virion. The invention further relates 10   to methods for the production of such RSV virions and to their use in vaccines and methods for vaccination against pneumoviruses.

Background of the invention

         Human respiratory Syncytial virus is classified in the genus Pneumovirus, family 15   Paramyxoviruses. It is a major cause of severe lower respiratory tract disease in infants, the elderly and in immunocompromised individuals. It is also an important factor in upper respiratory tract disease in older children and adults. Currently there is no effective h-RSV vaccine available in the art.

         RSV is an enveloped RNA virus that expresses two major antigens at its surface: 20   the attachment protein G and the fusion protein F. Both proteins appear to invoke protective antibodies. G is the determinant of the two known h-RSV subgroups A and B. Antigenic differences can be found within the two groups. The G protein shows a high degree of variation with only 53 % amino acid homology between groups A and B and up to 20% differences in G protein sequences within group A (Mufson 1988, Cane 25   1991).

         Passive immunisation with RSV-enriched immunoglobulin (Respigam) or synthetic humanised monoclonal antibodies against F (Palivizumab) is currently used to treat and protect neonates of certain predispositions (e.g. premature birth) against RSV infection (Robinson 2000, Greenough 2000). RSV pathology has two major 30   aspects: cell damage caused by the virus itself and tissue damage caused by the overreacting immune system. The latter is a highly complicating factor in vaccine design.

RSV infections are seasonal, limited to the winter period and peak in the Northern Hemisphere around the end of the year. RSV infects every child before the age of two, in many cases twice. Older individuals on average are infected every other year, depending on the setting; people in close contact with infants and young children

5   have a 50% risk. The virus spreads by close contact, in droplets or through contaminated surfaces. RSV is not efficiently spread through aerosols; the virus particles are relatively unstable. Internal spread of the virus from the upper respiratory tract (URT) to the lower respiratory tract (LRT) occurs predominantly by inhalation of virus particles produced in the URT epithelium during primary infection. Spread

10   through syncytium formation (one of the pathological properties of the virus, which gave it its name) can not be ruled out and may play a secondary role in LRT infection.

In general, RSV pathology starts in the URT; the port of entry is the nose and to a lesser extent the eyes – not the mouth. When restricted to URT tissues, disease is limited to common cold, although in adults sometimes severe. However, when the virus

15   can reach the LRT, bronchiolitis and pneumonia can ensue in unprotected individuals. In young infants, this can be life threatening, approx. 1/100 will require hospitalisation and mechanical ventilation, out of these 1% may die. In the elderly, RSV-induced LRT disease is a major cause of hospitalisation; it is suspected that RSV causes 25% of flu-like diseases.

20   The immune response to RSV is complex. In general, exposure to h-RSV will build up a response that protects against LRT disease. This response wanes with older age, causing the higher susceptibility to RSV of the older population. Effective long lasting protection against URT disease appears not feasible: re-infection is very common, even within the same season and this is not caused by viral variation.

25   Protection against RSV infection involves antibodies against viral proteins F and G circulating in the blood, which can prevent LRT disease. URT infection can be controlled by mucosal antibodies against F and G, but these have a limited life span. CD8+ T cells against as yet unidentified viral proteins are required to clear the virus from infected tissues, but they appear to be short-lived or inefficiently recruited from

30   their reservoirs. Most likely, this is caused by RSV-expressed factors, possibly encoded in the G gene (Srikiatkhachorn, 1997a).

An important aspect of RSV disease is immune enhancement of pathology. In limited cases the cellular immune response can exacerbate RSV disease by the action of

cytokines on infected tissues released from excessively attracted granulocytes. Host predisposition is involved in this reaction, but possibly also the timing of first RSV infection after birth. Unfortunately, early vaccine trials with formalin-inactivated RSV showed that in these vaccination settings immune enhanced pathology upon wt infection was prevalent (Kim 1969). Factors contained in RSV appear to be responsible for this phenomenon and were apparently released by formalin treatment. In the 40 years since then, it was gradually shown that the viral G protein is the predominant mediator of these problems, but the mechanism remains unclear (Srikiatkhachorn 1997b). In any case, vaccination with a G protein out of the context of the virion (i.e. in inactivated virus preparations, as expression product not properly embedded in a membrane or in the form of peptides) seems to be causing immune enhancement in model systems. Thus, although G contributes to some extent to RSV immunity, its properties also complicate vaccine design.

Initial live RSV vaccine candidates included cold passaged or temperature-sensitive mutants. The former have been attenuated by culturing at decreasing temperature, leading to dependency on low temperatures for growth, whereas the latter mutants have been made dependent on a specific, usually higher temperature for replication by chemical or radiation mutagenesis. These live virus vaccine candidates appeared to be either under- or overattenuated (Crowe 1998).

Subunit vaccine candidates are derived from either the RSV-F or the G protein, being the main targets for neutralising antibodies. A candidate subunit vaccine, PFP2, purified F protein, is safe in RSV-seropositive patients, but it did not provide full protection against LRT infection and associated disease (Gonzalez 2000). Another subunit vaccine approach is BBG2Na, which consists of a polypeptide, comprising amino acid 130-230 of h-RSV-G, fused to the albumin-binding domain of streptococcal G protein (Power 1997). BBG2Na induces a T helper type 2 response in neonatal mice, and does not elicit lung immunopathology (Siegrist 1999). There is no data yet on protection. The use of new adjuvants for a balanced humoral and cellular immune response are currently under investigation in animal models (Plotnicky 2003).

The use of plasmid-DNA vectors encoding RSV-F and G antigens as vaccine candidates has been studied in animal models. These vaccines induce protective responses in rodents (Li 2000), but in one study RSV-F DNA vaccine candidate immunised mice developed a slightly enhanced pulmonary inflammatory response

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following challenge with wt virus (Bembridge 2000). The feasibility of the use of plasmid DNA vaccines in humans is not yet known and it will likely take at least 15 years before this approach is sufficiently studied and - more importantly - accepted, particularly for neonates. Candidate vaccines based on vector delivery systems are

5 constructed of live recombinant vectors expressing RSV proteins. For example, recombinant vaccinia virus expressing RSV-F and G provided protection in mice, but lacked this effect in chimpanzees (Collins 1990). The question is whether these systems are safe (notably vaccinia virus) and feasible in the light of existing (maternal) antibodies against poxviruses in the community and the main target group being

10 neonates.

Several vaccine candidates are based on recombinant live RSV, generated by reverse genetics. One line of study focuses on attenuating these viruses by introducing the individual or combined mutations responsible for cold-adaptation and temperature-sensitivity into the recombinant virus. None of these vaccine candidates were usable,

15 because of either over- or underattenuation. Another line of study focuses on deletion of one or more viral non-structural genes. Limited data are available on the behaviour of these viruses in model systems (Jin 2003).

An alternative approach to RSV vaccine development is the use of bovine RSV. A chimeric bovine RSV with either the human F protein alone or both the human F and

20 G protein was evaluated for its efficacy in chimpanzees. This vaccine candidate was restricted in replication to such a degree that animals were not protected after wild type h-RSV challenge (Buchholtz 2000).

Thus, currently there is no effective h-RSV vaccine available in the art. All RSV vaccine candidates that have been tested in animal models are unusable in humans.

25 There is thus a long felt need in the art for RSV vaccines that are both effective and safe and it is an object of the present invention to provide for such vaccines.

Description of the invention

Definitions

30 In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one

of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

The term "virion" as used herein refers to a virus particle that contains the nucleocapsid protein, the viral genome and the replicase complex in a lipid envelop that contains the viral structural glycoproteins.

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The terms "infectivity of a virus", "infectious virus", "infectious virus particle" or "infectious virion" denote viruses, virus particles or virions that are capable of entering suitable host cells and initiating a virus replication cycle, whether or not this leads to the production new virus that is infectious.

10

Detailed description of the invention

In a first aspect the present invention relates to a virion of a pneumovirus. The virion comprises a viral genome that has a mutation in a gene coding for a protein that is essential for infectivity of the pneumovirus, whereby the mutation causes a virus

15 produced from only the viral genome to lack infectivity, and whereby the virion comprises the protein in a form and in an amount that is required for infectivity of the virion.

The pneumovirus preferably is a Respiratory Syncytial Virus (RSV), more preferably a human or bovine RSV. The human RSV may either be a subgroup A or B

20 virus. Therefore, any RSV strain or isolate may be used in the context of the present invention, whereby is understood that the invention is only exemplified by means of the particular human RSV isolate 98-25147-X, referred to as RSV isolate X.

The viral genome comprises at least one mutation in at least one viral gene coding for a protein that is essential for infectivity of the pneumovirus, whereby the

25 infectivity of the virus is as defined above. Thus, the protein that is essential for infectivity of the pneumovirus is a protein that is essential for the capability of the virion of the invention to enter a suitable host cell and initiate a viral replication cycle, whereby the replication cycle does not necessarily lead to the production of new infectious virions. In preferred virions of the invention the mutation causes the virion to

30 lack infectivity *in vivo*, i.e. in a suitable host organism, whereby the virions may still be infectious for suitable host cells cultured *in vitro*.

In a preferred virion of the invention, the mutated gene that codes for a protein essential for infectivity of the pneumovirus, is a gene, which codes for a structural

protein of the virus. A structural protein of a pneumovirus is herein understood to be a protein that is present in virions of wild-type infectious virus. Preferred genes coding for structural proteins to be mutated in the virions of the invention are the genes coding for the attachment protein G and/or the fusion protein F, whereby the G protein is most

5   preferred. Deletion and/or functional inactivation the gene coding for G protein serves several purposes and prevents a number of problems and complications of current RSV vaccine candidates. One purpose is vaccine safety: RSV without G protein is highly attenuated in its host (Karron 1997, Schmidt 2002) because it will not be able to efficiently infect host cells. One complication is that the G protein is strongly

10   implicated in causing undesired immunological responses, including enhanced immune pathology (Alwan 1993, Srikiatkhachorn 1997b) and possible skewing of the immune system towards an allergy (and asthma-) prone state under certain genetic predispositions (Openshaw 2003, Peebles 2003). This will be prevented by deletion or inactivation of the G gene. A pneumoviral virion of the invention comprising a viral

15   genome that has an inactivating mutation in the gene coding for a G attachment protein, and comprising the G attachment protein in a form and in an amount that is required for infectivity of the virion is referred to as a "ΔG+G" (pneumo)virus or virion. Similarly, the virion that has the inactivating mutation in the gene coding for a G attachment protein, but which is not complemented *in trans* with a functional amount of G protein

20   is referred to as a "ΔG" (pneumo)virus or virion.

In the virions of the invention, the mutation in the gene of the essential structural protein is a mutation that causes the virus produced from only the viral genome to lack the protein or to express a biologically inactivated protein. Production of virus from only the viral genome is understood to mean virus produced exclusively from the viral

25   genome as present in the virions and in the absence of any coding sequence complementing the viral genome *in trans*. The viral genome as present in the virions is thus incapable of directing expression of the essential structural protein. This may be achieved in various ways known to the skilled person, including e.g. inactivation of the translation initiation codon, introduction of stop codons near the N-terminus of the

30   encoded protein, one or more frame-shift mutations, deletion of one or more fragments from the gene. Most preferred is however, a virion in which the mutation comprises deletion of the (entire) sequence coding for the protein.

Explicitly included in the invention are virions in which more than one mutation is present. In particular, more than one viral protein-coding gene may comprise mutations that inactivate or alter the function of the protein in question, or which cause the protein to lack from the virions as described above. E.g. the cold-passaged or heat-

5   sensitive mutations as known in the art may be combined with inactivation of the essential structural proteins as disclosed in the invention above.

Clearing of pneumoviruses like RSV from the infected host organisms requires proper cellular immunity, which will not be effectively mounted without infection of epithelial cells by the virus. However, the mutant pneumoviruses of the invention lack

10   genetic information for a protein that is essential for infection of host cells *in vivo*. Therefore the present invention discloses methods for the production of the mutant pneumoviruses, which include replication of mutant pneumoviruses in cells that complement (*in trans*) for the absence of the protein that is essential for infection.

In another aspect the invention thus pertains to a method for producing the above

15   defined mutant pneumoviral virions. The method is a method for producing pneumoviral virions, whereby the virions comprise a viral genome that has a mutation in a gene coding for a protein that is essential for (in vivo) infectivity of the pneumovirus, whereby the mutation causes a virus produced from only the viral genome to lack infectivity, and whereby the virion comprises the protein in a form and

20   in an amount that is required for infectivity of the virion. The method comprises the steps of: (a) infecting a culture of a first host cell with a pneumovirus comprising a viral genome that has a mutation as defined above, whereby the host cell comprises an expression vector which directs expression, either transiently or constitutively, in the host cell of the protein in a form and in an amount that is required for infectivity of the

25   virion; and, (b) recovery of the virions from the infected host cell culture. Recovery of virions from the infected host cell culture may include either or both recovery from the culture medium as well as recovery from the cells.

The first host cell may be any host cell in which the pneumovirus is capable of replication, with or without the simultaneous expression *in trans* of the protein that is

30   required for infectivity of the virion. Suitable host cells for this purpose are e.g. African green monkey kidney cell cultures (such as e.g. Vero, ECACC lot 10-87, 134th passage, 1990, EMEA approved).

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In a preferred method of the invention, the pneumovirus that is used to infect the culture of a first host cell culture, is produced in a method comprising the steps of: (a) providing to a second host cell one or more expression vectors which direct expression in the host cell of: (i) a viral genomic RNA that has a mutation in a gene coding for a
5   protein that is essential for (in vivo) infectivity of the pneumovirus, whereby the mutation causes a virus produced from only the viral genome to lack infectivity; and, (ii) a pneumoviral polymerase enzyme complex and optionally one or more further viral proteins; and, (b) culturing the second host cell whereby the virions are produced. In a preferred method, the virions produced by the second host cell are amplified by
10   one or more further cellular infection steps employing host cells which are the same or different from the second host cell.

The second host cell may be any host cell in which the pneumovirus is capable of replication, with or without the simultaneous expression *in trans* of the protein that is required for infectivity of the virion. Suitable host cells for this purpose are e.g. African
15   green monkey kidney cell cultures (such as e.g. Vero, ECACC lot 10-87, 134th passage, 1990, EMEA approved), or Hep-2 cells. The second host cell may be the same as or different from the first host cell.

In the methods of the invention, the viral genomic RNA is transcribed from a viral DNA copy that is under the control of a bacteriophage DNA-dependent RNA
20   polymerase promoter and whereby the (second) host cell is provided with an expression vector which directs expression in the host cell of the bacteriophage DNA-dependent RNA polymerase. Preferably, the bacteriophage DNA-dependent RNA polymerase is a T7, T3 or SP6 polymerase.

The pneumoviral polymerase enzyme complex that is expressed from one or
25   more expression vector(s) in the second host cell at least includes the L, P, N proteins expressed from their corresponding genes or cDNA's in the expression vector(s). For improved efficiency of viral assembly and packaging of the naked viral genomic RNA, optionally, one or more further viral proteins are expressed in the second host cells. Preferred viral proteins for this purpose include the viral matrix membrane proteins of
30   which the M2-1 protein is particularly preferred. The L, P, N, M2-1, G or F proteins are preferably derived from the viral genome of the viral isolate which is introduced and expressed in the host cell, but alternatively also homologous proteins from other heterologous viral or non viral sources may be used.

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The skilled person will appreciate that a wide variety of expression vectors and regulatory sequences (such as promoters) are available in the art for expression of the viral genomic RNA, the DNA-dependent RNA polymerase, pneumoviral polymerase enzyme complex and optional further viral proteins, as well as the essential structural
5   protein, in the first and/or second host cells (see e.g. Sambrook and Russell (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York).

For reverse genetics of RNA viruses, i.e. expression of a recombinant RNA virus such as the virions of the present invention, a cDNA copy of the viral genomic RNA is
10 cloned into plasmids and is placed under the control of sequences that will allow synthesis of RNA from the DNA under certain conditions. Generally, the promoter sequence for bacteriophage RNA polymerase (e.g. the T7 RNA polymerase) is placed upstream of the DNA copy of the RNA genome, while an appropriate terminator for the RNA polymerase is placed downstream of the genome. Self-cleaving ribozyme
15 sequences are placed upstream of the terminator sequences, to allow synthesis of RNA with the correct terminal nucleotides. Correct terminal sequences are generally required to rescue virus from the synthetic RNA. For non-segmented negative strand RNA viruses, co-expression of the polymerase enzyme complex (N, P and L proteins for Paramyxoviruses) along with the genomic or anti-genomic RNA is required to obtain
20 recombinant virus (reviewed by Neumann 2002 and exemplified in the Examples herein).

Other preferred methods may comprise the further step of isolating and/or purifying the virions of the invention and/or formulating these virions into pharmaceutical compositions. Methods for isolating and/or purifying virions are well
25 known to the skilled virologist. Such methods e.g. include various centrifugation techniques (e.g. differential or density centrifugation), or chromatographic techniques. A method for formulating the virions of the invention into a pharmaceutical composition at least comprises the step of mixing the virions with a pharmaceutically acceptable carrier as defined below.

30   In a further aspect the invention relates to a composition comprising a virion as defined above or obtainable in a method as defined above, and a pharmaceutically acceptable carrier. The composition preferably is a pharmaceutical composition that is preferably suitable for use as a vaccine, i.e. the composition preferably is a vaccine.

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In a yet another aspect the invention provides for a pharmaceutical preparation comprising as active ingredient a virion according to the invention, and a pharmaceutically acceptable carrier. Pharmaceutically acceptable stabilising agents, osmotic agents, buffering agents, dispersing agents, and the like may also be

5   incorporated into the pharmaceutical compositions. The preferred form depends on the intended mode of administration and therapeutic application. The pharmaceutical carrier can be any compatible, non-toxic substance suitable to deliver the reconstituted viral membranes to the patient. Pharmaceutically acceptable carriers for intranasal delivery are exemplified by water, buffered saline solutions, glycerin, polysorbate 20,

10   cremophor EL, and an aqueous mixture of caprylic/capric glyceride, and may be buffered to provide a neutral pH environment.

For administration by inhalation, the pharmaceutical compositions of the present invention are conveniently delivered in the form of an aerosol spray from pressurised packs or a nebuliser, wherein the virions are present in a carrier as described for

15   intranasal delivery but with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurised aerosol the dosage unit may be determined by providing a valve to deliver a metered amount.

Methods for preparing intranasal or inhalant compositions are well known in the

20   art and described in more detail in various sources, including, for example, Remington's Pharmaceutical Science (15th ed., Mack Publishing, Easton, PA, 1980) (incorporated by reference in its entirety for all purposes). The virions may thus be formulated as the active component in any preparation for vaccination, which may further e.g. include carriers, adjuvants, stabilisers, solubilisers, preservatives and other

25   excipients known in the art, to allow or to aid efficient administration of the preparation for vaccination to individuals, preferably human and live stock or farm animals (such as cows, pigs, horses, goats, sheep).

In a further aspect, the invention relates to a method for vaccination against, or for prophylaxis or therapy (prevention or treatment) of an pneumoviral infection by

30   administration of a therapeutically or prophylactically effective amount of (a pharmaceutical composition comprising) the virions of the invention as defined above, or obtainable as defined above, to a subject in need of prophylaxis or therapy. Preferably, the virions are administered intranasally.

The invention similarly relates to virions of the invention as defined above, or obtainable as defined above, for use as a medicament, preferably a medicament for vaccination against, or for prophylaxis or therapy of a pneumoviral infection. The invention further relates to the use of the virions of the invention in the manufacture of

5 a medicament for vaccination against, or for prophylaxis or therapy of a pneumoviral disease or infection. Preferably the medicament is a preparation for intranasal administration.

The compositions comprising the virions of the invention for vaccination are preferably administered intranasally to appropriate hosts. In one embodiment, calves

10 are to be protected from b-RSV infections. In yet another embodiment, humans, of which preferably infants and elderly or immune compromised individuals are protected from h-RSV infections. Formulations preferably comprise formulations suitable for administration as intranasal drops or spray, preferably a nasal spray. The ΔG+G-pneumoviral particles in the composition will infect epithelial cells of the upper

15 respiratory tract only once because the second generation virions produced from the initially infected URT epithelial cells lack the G attachment protein for which the coding sequence has been removed from the genome. These ΔG-virions are therefore non-infectious *in vivo* in host organisms. However, the initial single cycle of infection allows for the development of appropriate cellular immunity - that is a response capable

20 of clearing wild-type virus infection - to be mounted against pneumovirus, or RSV in particular, while protective antibodies against F - i.e. antibodies that will prevent lower respiratory tract infection - will be elicited by the vaccine and the non-infectious progeny. Anti-F antibodies are effective in limiting RSV infection, as is shown by the effectiveness of Palivimuzab treatment, which is a humanised monoclonal antibody

25 against F. This is the basis of the efficacy of the recombinant live attenuated pneumoviral vaccines of the invention. These live viral vaccines solves a number of problems associated with current pneumovirus vaccine candidates. The presence of the G-protein in its natural context in the virion allows for the development of appropriate cellular immunity whereas the undesirable effects of immunity against the isolated G

30 protein that is largely responsible for immune enhancement of b-RSV and h-RSV pathology in cattle and humans respectively, is avoided.

Description of the figures

Figure 1: Diagram of construction of pRSVXΔG. Upper line represents RSV isolate X genomic RNA, with genes indicated. Boxes below represent RT-PCR products and oligonucleotide duplexes used for construction. Numbers inside boxes indicate the oligonucleotide numbers as listed in table I. Restriction sites introduced for cloning are

5   indicated. The final cloning scheme is indicated below: circles are plasmids and the arrows show the order of cloning.

Figure 2. Alignments showing the differences between RSV isolate X and pRSVXΔG sequences. Sequences are shown as alignment of genomic sense. For pRSVXΔG only nucleotides differing from RSV isolate X are indicated. Similar sequences are indicated

10   by dots (.) and gaps are indicated by (-). Gene start signals are single underlined, gene stop signals double underlined, and the genes are indicated in the captions. Boxes outline the restriction enzyme recognition sites resulting from the nucleotide changes introduced.

Table I. Primers used for RT-PCR cloning of RSV isolate X.

15   Table II. Primers used for cloning of helper plasmids and for plasmids used for construction of stable cell lines.

Examples

The current invention is illustrated by the following non limiting examples that are merely used to illustrate specific embodiments of the invention and should not be read as limiting the general scope or any aspect of the invention.

5

Example 1

Viral isolate, virus isolation, propagation and storage

The basis for the recombinant h-RSV clone is a clinical RSV isolate, obtained from the Leiden University Medical Centre diagnostic laboratory. This virus, named 98-25147-X, coded after the patient from which it was isolated, was derived from a diagnostic test on Hep-2 cells in the period 21-24 December 1998. It was later determined to be a subtype A isolate and is designated RSV isolate X. The virus was passaged 4 times on Hep-2 cells in T75 bottles in DMEM (Gibco), 10% FCS, pen/strep/glu and subsequently five times on Vero cells in T75 bottles on in DMEM (Gibco), 10% FCS, pen/strep/glu. The resulting RSV isolate X virus was used as working stock and stored at -135^0C in 25% or 45% sucrose.

Example 2

Construction of RSV-X cDNA encoding viral genome

Total RNA was obtained by phenol-guanidine isothiocyanate extraction (Trizol, Invitrogen) of stock RSV isolate X infected Vero cells. cDNA was prepared by reverse transcription using Thermoscript (Invitrogen) reverse transcriptase using random hexamer primers. This cDNA was used as template for PCR using High fidelity Taq polymerase (Invitrogen) using specific primers containing restriction enzyme recognition sites (Table I and sequence listing). Primers were designed based on the published sequences of RSV-A2 (Genbank accession no M74568) and RSV-RSS2 (Genbank accession no U39662).

PCR products were first cloned individually in different vectors: primer pairs, vectors, restriction enzyme recognition sites and resulting vector name are listed below.

RSV021/RSV047: pCAP vector (Roche), bluntly into Mlu N1, pCAP3 (SH/M/P region)

RSV018/019: pCAP vector, bluntly into Mlu N1, pCAP2 (G region)

RSV016/RSV017: PUC21, Mlu I /Bam HI, pUK5 (M2-2/M2-1/F region)

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RSV024/RSV025a : PUC21, Bam HI/Afl II, pUK1 (NS2/NS1 region)

RSV022/ RSV023: PUC21, EcoR V, pUK4 (N region)

RSV014/ RSV015: PUC21, Kpn I/Mlu I, pUK2 (L region)

At least two individual clones derived from two independent cDNA templates

5 were sequenced; regions containing differences between the two clones were

sequenced on a third clone. If necessary, clones were repaired using standard molecular

biology techniques known by the skilled person. Additional PCR products covering the

binding sites of the primers used for cloning were obtained and sequenced. The 5'

genomic termini were determined by poly-adenylation of genomic RNA, followed by

10 RT-PCR with an oligo(d)T containing primer ALG018:

TTAAAAGCTTTTTTTTTTTTTTTTTTT

and an NS1 gene primer RSV126:

AATTCTGCAGGCCCATCTCTAACCAAAGGAGT.

This fragment was cloned into pUC21 using Hind III/Pst I. The 3'-end was determined

15 by RACE (rapid amplification of cDNA ends) ligation PCR. All sequences were

assembled to yield the RSV-X consensus sequence (Seq ID No. 1).

All sequences were confirmed by PCR cycle sequencing using the BigDye

terminator kit (Applied Biosystems) and analysed by an ABI Prism 310 genetic

analyser.

20

Example 3

Construction of ΔG-RSV isolate X full length plasmid

The full-length cDNA spanning the entire RSV isolate X genome was assembled

by sequential ligation of PCR fragments (Figure 1). The "trailer" end is preceded by the

25 promoter for the bacteriophage T7 polymerase. To generate correct 3' ends the cDNA

"leader" end is fused to the hepatitis delta virus ribozyme (HDVR), followed by a

terminator of the T7 RNA polymerase transcription (see Figure 1).

First, two sets of complementary oligomers encoding the HDVR and the T7

terminator RSV026/RSV027 oligo's and RSV028/029 oligo's were phosphorylated

30 with T4 DNA kinase, hybridised and ligated into clone pUK1 (containing genes

NS1/NS2) via Rsr II /Not I, giving plasmid pUK3. Then, the Xma I/SexA I fragment of

clone pUK4 containing N was ligated into plasmid pUK3 via Xma I/SexA I. This

15

plasmid (pUK6) contains the region from the N gene up to the 3' leader sequence, fused to the HDVR and a T7 terminator.

Secondly, the Xma I/Eco RV fragment of plasmid pCAP3 was inserted in plasmid pUK5 using Xma I and a filled-in Hind III site. This yields plasmid pUK8.

5   Subsequently, pUK 8 was digested with BssH II and BsiW I, ends were filled-in with Klenow polymerase and religated. This plasmid contains the genes M2-2, M2-1, F, SH, M and P and is named pUK9.

To synthesise a low-copy number vector for the RSV isolate X cDNA, two complementary oligomers, RSV011:

10   AGCTTGCGGCCGCGTCGACCCGGGACGCGTCGATCGGGTACCAT and RSV012: CGATGGTACCCGATCGACGCGTCCCGGGTCGACGCGGCCGCA were phosphorylated with T4 DNA kinase, hybridised and inserted in the alkaline phosphatase treated and Cla I /Hind III digested plasmid pACYC184 (New England Biolabs). The resulting plasmid is named pACYC184-MCS. Subsequently a Mlu I-

15   Knp I fragment of pUK2 containing the T7 promoter and L gene was inserted, this intermediate plasmid is named pACYC1. Then, the region from the N gene up to the 3'-leader sequence, including the fused HDVR and T7 terminator sequence of pUK6 was added to pACYC1 using Xma I/Not I. This gives intermediate plasmid pACYC2. Finally, the Xma I /Mlu I fragment of pUK9 containing the M2-2, M2-1, F, SH, M and

20   P genes was inserted into pACYC2, yielding plasmid pACYC3, comprising the whole RSV genome of strain X lacking the G gene. Sequence analysis of the latter plasmid revealed a deletion in the HDVR region, which was repaired and the resulting plasmid is named pRSVXΔG.

In addition to construct pRSVXΔG, construct pACYC24 was generated in which

25   the genomic RSV isolate X insert is reverse complemented via inverse PCR. From the construct, antigenomic RSV RNA can be synthesised. In pACYC24, the T7 promoter precedes the 3'-leader sequence, whereas the HDVR and T7 terminator are fused to the 5'-trailer sequence.

All restriction enzyme recognition sites used to construct pRSVXΔG are located

30   inside the RSV intergenic regions and do not alter coding sequences or affect transcription signals (as shown in Figure 2).

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Example 4

Construction of helper plasmids

Helper plasmids expressing several RSV proteins were constructed as follows.
All required genes are derived from lab-strain RSV-A2 (ATCC #VR1302). Virus was

5   plaque-purified on Hep-2 cells and subsequently used to infect Vero cells. Total RNA
was isolated from these cells by phenol-guanidine isothiocyanate extraction (Trizol,
Invitrogen) and subjected to RT-PCR using High Fidelity Taq polymerase (Invitrogen)
and a set of primers specific for RSV genes L, P, N and M2-1 respectively (see Table
II). PCR products were subsequently cloned into expression plasmids pcDNA3,

10   pcDNA6 or pCI, using restriction enzyme recognition sites as indicated in the table II.
Clone sequences were confirmed by PCR cycle sequencing using the BigDye
terminator kit (Applied Biosystems) and analysed by an ABI Prism 310 genetic
analyser.

15   ## Example 5

Construction of G-producing Vero cell-lines

Cell lines producing RSV-G protein were constructed using several methods:
In method 1, the G gene from either RSV-A2 or RSV isolate X, or the G gene
from RSV-A2, in which the internal translation initiation codon had been disabled by

20   modification using primers RSV033 and RSV 034, were cloned into expression vector
pcDNA3 or pcDNA6 (Invitrogen) using RT-PCR on RNA from RSV-A2 or RSV
isolate X infected Vero cells using primers as indicated in Table II. The plasmids were
introduced into Vero cells using either chemical agents $CaCl_2$, co-precipitation,
liposome-based or electroporation (Ausubel 1989). Two methods for isolating stable

25   cell lines were used. In the first method, 72 hours after transfection, cells were split
using various dilutions into fresh medium containing selective medium, zeocin for
pcDNA3 and blasticidin for pcDNA6. Cells were fed with selective medium every 3-4
days until cell foci were identified. Single colonies were picked and transferred in to
96-well plate, or seeded in various dilutions to obtain single cells in a 96 well plate.

30   Antibiotic resistant colonies were tested on expression of RSV-G by immunostaining
techniques or FACS using RSV G-specific antibodies. Colonies expressing G were
passaged, and were designated as stable cell lines expressing G. The second method
comprises FACS sorting using RSV-G specific antibodies 72 hours after transfection.

RSV-G expressing cells were seeded in a serial dilution to obtain single cells in a 96-well plate and cultured with selective medium. Single cell colonies were passaged on selective medium and subsequently tested again for expression of RSV-G, resulting in cell lines expressing RSV-G.

5   In method 2, the Flp-In system (Invitrogen) is used to produce Vero cells with target gene insertion sites at chromosomal positions which allow different levels of target gene expression. The RSV-G gene, derived from the plasmids from method 1 but with a modification (introduced using primer RSV151: Table II) of the G translation initiation codon surrounding sequence to allow higher translation levels, were inserted

10   in each of these cell lines using the system-generic method, resulting in Vero cell lines stably expressing different levels of G protein.

      In method 3, Vero cells were transiently made to express the G protein, by either transfection with the expression plasmids containing the G gene from method 1, or by infection with Modified vaccinia virus Ankara (MVA) (Sutter 1992) or fowlpox viruses

15   (Spehner 1990) expressing the G protein.

Example 6
Construction of bacteriophage T7-polymerase-producing cell lines

      The bacteriophage T7 polymerase gene is PCR amplified from plasmid pPRT7

20   (van Gennip 1997), containing the gene, using primers ALG022 and ALG023 (Table II). The PCR product is cloned into pcDNA6b vector, using Hind III/Xba I, yielding plasmid pc6T7pol. Vero cells were transfected using lipofectamine 2000 as recommended by the manufacturer (Invitrogen). 72 hours after transfection cells were split and grown in fresh medium containing blasticidin. Cells were fed fresh medium

25   every 3-4 days and split twice to obtain larger culture volumes. 20 days after transfection, blasticidin resistant cells were transfected with reporter plasmid pT7-IRES2-EGFP using lipofectamine 2000. For the construction of plasmid pT7-IRES2-EGFP, first plasmid pT7-EGFP was constructed by inserting via HindIII/BamH1 in plasmid p-EGFP-N1 (Clonetech) a set of complementary oligomers encoding for the

30   T7 promoter sequence (ALG32: AGCTAATACGACTCACTATAGGGAGACGCGT and ALG33: GATCACGCGTCTCCCTATAGTGAGTCGTATT). Plasmid pT7-IRES2-EGFP was then constructed by cloning the T7-EGFP fragment of plasmid pT7-EGFP into plasmid p-IRES2-EGFP via Xma1-Not1. Cells expressing EGFP were

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sorted by FACS and grown in limited dilution to obtain single cell colonies. Single colonies expressing T7 RNA polymerase were tested for stability, grown to larger culture volumes and stored.

5   Example 7

Method to produce recombinant ΔG-RSV isolate X virus

Hep-2 cells were cultivated in DMEM + 10% FCS (foetal calf serum) + penicillin/ streptomycin/ glutamine, whereas Vero cells and derivatives thereof are cultivated in M199 + 5% FCS + pen/strep/glu. Cells were grown overnight to 80%

10   confluence in 10 mm² dishes at 37⁰C. For Vero and Hep-2 cells, cells were infected with modified virus Ankara-T7 (MVA-T7)(Sutter 1992, Wyatt 1995) or fowlpox-T7 virus (Britton 1996) at MOI = 3 (multiplicity of infection 3) and incubated at 32⁰C for 60 min prior to transfection, to allow expression of bacteriophage T7 polymerase. The cells (Hep-2, Vero or Vero-T7 cells) were washed with Optimem medium (Optimem 1

15   with glutamax, Invitrogen) and subsequently transfected with helper plasmids encoding the N, P, L and M2.1 genes of RSV and with plasmid pRSVXΔG, using Lipofectamine2000 (Invitrogen) in Optimem (total volume 500 μl). The following amounts of plasmids were added: 1.6 μg pRSVXΔG, 1.6 μg pcDNA6-A2-N, 1.2 μg pcDNA3-P, 0.4 μg pcDNA6-A2-L, 0.8 μg pcDNA6-A2-M2.1. After 3-4 hrs of

20   incubation at 32⁰C, 500 μl of Optimem medium with 2% FCS was added and the cells were incubated at 32⁰ C for 3 days. Cells were then scraped and the mixture of scraped cells and medium containing the rescued virus was used to infect fresh cultures of Vero or Hep-2 cells grown in DMEM + 2% FCS + pen/strep/glu. The latter procedure was repeated for 4-5 times to obtain high titre virus stocks.

25

                                     Example 8

Method to produce recombinant ΔG+G-RSV isolate X virus

ΔG-RSV isolate X virus, derived from transfected Vero cells, was passaged several times to obtain titres of at least 10^5 pfu/ml (plaque forming units per ml).

30   Different moi's of this virus were then used to infect the Vero cell line producing RSV-G protein. The resulting ΔG+G-RSV isolate X was harvested from the medium and/or from the cells and analysed for the presence of the G protein in the virions by immunodetection techniques. Infectivity titres were determined on Vero or Hep-2 cells,

19

and the integrity of the ΔG-genome was determined using RT-PCR on viral RNA extracted from cells infected with ΔG+G-RSV isolate X virus. Virus was stored at — 135⁰ C in 25% or 40% sucrose.

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Claims

1.   A virion of a pneumovirus comprising a viral genome that has a mutation in a gene coding for a protein that is essential for infectivity of the pneumovirus, whereby

5   the mutation causes a virus produced from only the viral genome to lack infectivity, and whereby the virion comprises the protein in a form and in an amount that is required for infectivity of the virion. ,

2.   A virion according to claim 1, whereby the pneumovirus is a Respiratory

10   Syncytial Virus.

3.   A virion according to claims 1 or 2, whereby the gene codes for a G attachment protein.

15   4.   A virion according to any one of claims 1-3, whereby the mutation causes the virus produced from only the viral genome to lack the protein.

5.   A virion according to claim 1, whereby the mutation comprises deletion of the sequence coding for the protein.

20

6.   A method for producing pneumoviral virions, the virions comprising a viral genome that has a mutation in a gene coding for a protein that is essential for (in vivo) infectivity of the pneumovirus, whereby the mutation causes a virus produced from only the viral genome to lack infectivity, and whereby the virion comprises the protein

25   in a form and in an amount that is required for infectivity of the virion, the method comprising the steps of:
(a) infecting a culture of a first host cell with a pneumovirus comprising a viral genome that has the mutation, whereby the host cell comprises an expression vector which directs expression in the host cell of the protein in a form and in an amount that is

30   required for infectivity of the virion; and,
(b) recovery of the virions from the infected host cell culture.

7.   A method according to claim 6, whereby the pneumovirus that is used to infect the culture of a first host cell culture, is produced the method comprising the steps of:

(a) providing to a second host cell one or more expression vectors which direct expression in the host cell of:

5   i) a viral genomic RNA that has a mutation in a gene coding for a protein that is essential for (in vivo) infectivity of the pneumovirus, whereby the mutation causes a virus produced from only the viral genome to lack infectivity;

ii) a pneumoviral polymerase enzyme complex and optionally one or more further viral proteins; and,

10   (b) culturing the second host cell whereby the virions are produced.

8.   A method according to claim 7, which further comprises amplifying the virions produced by the second host cell by one or more further cellular infection steps employing host cells which are the same or different from the second host cell.

15

9.   A method according to claims 7 or 8 wherein the viral genomic RNA is transcribed from a viral DNA copy that is under the control of a bacteriophage DNA-dependent RNA polymerase promoter and whereby the host cell is provided with an expression vector which directs expression in the host cell of the bacteriophage DNA-

20   dependent RNA polymerase.

10.   A method according to claim 9 whereby the bacteriophage DNA-dependent RNA polymerase is the T7, T3 or SP6 polymerase.

25   11.   A method according to any one of claims 7-10, whereby the pneumoviral polymerase enzyme complex at least includes the L, P, N proteins.

12.   A method according to any one of claims 7-11, whereby one or more further viral proteins is a pneumoviral matrix membrane protein, preferably the M2-1 protein.

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13.   A method according to any one of claims 6-12, whereby the pneumovirus is a Respiratory Syncytial Virus.

14. A method according to any one of claims 6-13, whereby the gene coding for the protein that is essential for infectivity is a gene coding for a G attachment protein.

15. A composition comprising a virion as defined in any one of claims 1-5, or obtainable in a method as defined in any one of claims 6-14, and a pharmaceutically acceptable carrier.

16. Use of a virion as defined in any one of claims 1-5 for the manufacture of a medicament for the prevention or treatment of a pneumoviral infection.

17. A use according to claim 16, the medicament is a preparation for intranasal administration.

18. A method for the prevention or treatment of a pneumoviral infection, the method comprising the step of administering to a subject a composition comprising a virion as defined in any one of claims 1-5, in an amount effective to prevent or treat the infection.

19. A method according to claim 18, wherein the composition is administered intranasally.

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Abstract

The present invention relates to pneumoviral virions comprising a viral genome that has a mutation in a gene coding for a protein that is essential for infectivity of the pneumovirus, whereby the mutation causes a virus produced from only the viral

5   genome to lack infectivity, and whereby the virion comprises the protein in a form and in an amount that is required for infectivity of the virion. The invention for relates to methods for producing the pneumoviral virions and for using the virions in the treatment or prevention of pneumoviral infection and disease. A preferred pneumoviral virion is a virion of Respiratory Syncytial Virus in which preferably the gene for the G

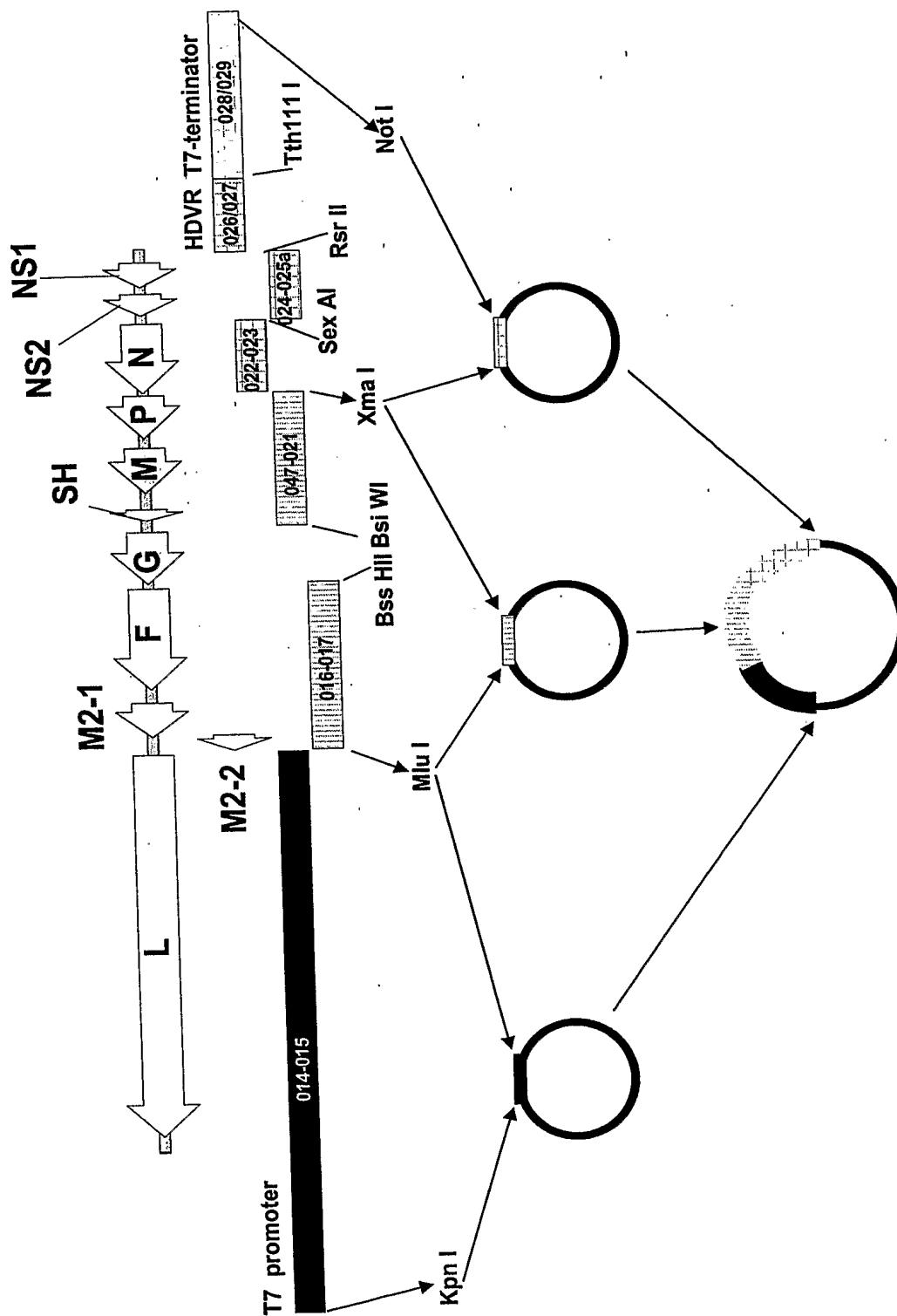10   attachment protein is inactivated and complemented *in trans*.

Figure 1.

FIGURE 2

Intergenic region between L and M2-2

RSV-X   ATTTTGTCCCATAGCTTGAATTGTTTGAGTTAATAGTTTGATGATGGTGGTAAGCATTAGGATTGAGTGTTA

pRSVXΔG   .... | C.C.T | ....

Intergenic region between P and N

RSV-X   AAACTTTTCCATGATGATTATTTGCCCC---ACTTTTTTTATTAACT

pRSVXΔG   ....T.......| XmaI |...GGG............

Intergenic region between N and NS2

RSV-X   ATTTGCCCCATCTTTCATCTTAT--GTCTCTCCTTAATTTTAAATTACT

pRSVXΔG   ....| SexAI |.......CCAG...........

Region of G gene deletion

RSV-X   CTTTTTGCAATAATA---CGCTTTTTAATGACT | G gene | GTTTGCATTTGCCCCAATGT---TGTTGTTGGTCTTAATATTTTAGTTCATTGTTATGACTATTTTTAATTAACT

pRSVXΔG   ....| BssHII |.......GCG...----------C..ACG A....A........C.......

   | BsiWI |

Table I. Primers used for RT-PCR cloning of RSV isolate X

Template region	Primer name	Sequence
L	RSV014	AATTGGTACCTAATACGACTCACTATAGGGACGAGAAAAAAGTGTC
	RSV015	TTAAACGCGTCATCAAACTATTAACTC
M2-2/M2-1/F	RSV016	AATTACGCGTTAAGCATTAGGATTGAGTG
	RSV017	TTAAGGATCCGCGCGCTATTATTGCAAAAAGCC
G	RSV018	AATTGGCGCGCCTTTTAATGACTACTGG
	RSV019	TTAAGGATCCGTACGTTGGGGCAAATGCAAACATGTCC
SH/M/P	RSV021	TTAACCCGGGGCAAATAAAACATCATGG
	RSV047	AATTCGTACGTATTGTTAGTCTTAATATCTTAGTTCATTGTTATGA
N	RSV022	AATTCCCGGGATTTTTTTATTAACTCAAAGC
	RSV023	TTAAACCTGGTAAGATGAAAGATGGGGCAAATACAAAAATGGC
NS2/NS1	RSV024	AATTGGATCCACCAGGTCTCTCCTTAATTTTAAATTAC
	RSV025a	AATTCTTAAGGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCACGCGAAAAATGCGTACAAC
HDVR	RSV026	GTCCGACCTGGGCATCCGAAGGAGGACG
	RSV027	ACGTCCTCCTTCGGATGCCCAGGTCG
HDVR-T7phi	RSV028	TCGTCCACTCGGATGGCTAAGGGAATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTGC
	RSV029	GGCCGCAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATTCCCTTAGCCATCCGAGTGGACG

Table II. Primers used for cloning of helper plasmids and for plasmids used for construction of stable cell lines.

Gene	Primer name	Sequence	Restriction sites
L	RSV045	TTAACTCGAGTTATTCATTATGAAAGTTG	Xho I
	RSV046	AATTGGTACCGGGGACAAAATGGATCCC	Kpn I
P	RSV043	TTAATCTAGATTGTAACTATATTATATAG	Xba I
	RSV012a	AATTGGATCCGGGGCAAATAAATCATCATGG	BamH I
N	RSV010A	AATTGGATCCGGGGCAAATACAAGATGGC	BamH I
	RSV011	TTAACTCGAGATTAACTCAAAGCTCTACATC	Xho I
M2-1	RSV124	AATTGGATCCGGGGCAAATATGTCACGAAGG	BamH I
	RSV125	TTAATCTAGATCAGGTAGTATCATTATTTTGGC	Xba I
A2-G	RSV042	TTAATCTAGAAGTAACTACTGGCGTG	Xba I
	RSV004a	AATTGGATCCGGGGCAAATACAAACATGTCCAAAAACAAGGACC	BamH I
	RSV151	AATTCCATGGGGTCCAAAACCAAGGACCAACG	Nco I
A2-GΔM48	RSV033a	AAAAGTATACTTAATGTGATTGTGCTATAG	Acc I
	RSV034	TTTTGTATACTGGCAGCTATAATCTCAACTTCACTTATAATTGC	Acc I
X-G	RSV004a	AATTGGATCCGGGGCAAATACAAACATGTCCAAAAACAAGGACC	BamH I
	RSV018a	AATTTCTAGATTTTAATGACTACTGG	Xba I
T7pol	ALG022	TTAATCTAGACGTTACGCGAACGCGAAGTCC	Xba I
	ALG023	AATTAAGCTTACCATGGACACGATTAACATCGCTAAGAACG	Hind III